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3. Full name, address and postcode of the or of
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Bio/Gene Limited, BioGene House, 6 The Business Centre,
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733358600

Patents ADP number (if you know it)

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4. Title of the invention

DETECTION SYSTEM

5. Name of your agent (if you have one)

Stephen Skelton et al

"Address for service" in the United Kingdom
to which all correspondence should be sent
(including the postcode)

D/IPR
Poplar 2
MOD (PE) Abbey Wood # 19
Bristol
BS34 8JH
United Kingdom

Stephen Richard Skelton
D/IPR (DERA) Formalities
Poplar 2
MOD(PE) Abbey Wood/19
Stoke Gifford
BRISTOL BS34 8JH

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Number of earlier application

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Detection System

The present invention provides a method for detecting a target polynucleotide in a sample, for example by quantitatively monitoring an amplification reaction, as well as to probes and kits for use in these methods. The method is also suitable for the detection of polymorphisms or allelic variation and so may be used in diagnostic methods

10 Known fluorescence polymerase chain reaction (PCR) monitoring techniques include both strand specific and generic DNA intercalator techniques that can be used on a few second-generation PCR thermal cycling devices.

15 Generic methods utilise DNA intercalating dyes that exhibit increased fluorescence when bound to double stranded DNA species. Fluorescence increase due to a rise in the bulk concentration of DNA during amplifications can be used to measure reaction progress and to determine the target molecule
20 copy number. Furthermore, by monitoring fluorescence with a controlled change of temperature, DNA melting curves can be generated, for example, at the end of PCR thermal cycling.

When generic DNA methods are used to monitor the rise in bulk
25 concentration of nucleic acids can be determined without any time penalty. A single fluorescent reading can be taken at the same point in every reaction. End point melting curve analysis can be used to discriminate artefacts from amplicon, and to discriminate amplicons. Peaks of products can be seen
30 at concentrations that cannot be visualised by agarose gel electrophoresis.

In order to obtain high resolution melting data, the melt
experiment must be performed slowly on existing hardware
35 taking up to five minutes. However, by continually monitoring fluorescence amplification, a 3D image of the hysteresis of melting and hybridisation can be produced.

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This 3D image is amplicon dependent and may provide enough information for product discrimination.

- 5 It has been found that DNA melting curve analysis in general is a powerful tool in optimising PCR thermal cycling. By determining the melting temperatures of the amplicons, it is possible to lower the denaturing temperatures in later PCR cycles to this temperature. Optimisation for amplification from first generation reaction products rather than the
- 10 genomic DNA, reduces artefact formation occurring in later cycles. Melting temperatures of primer oligonucleotides and their complements can be used to determine their annealing temperatures, reducing the need for empirical optimisation.
- 15 The generic intercalator methods however are only quasi-strand-specific and therefore is not very useful where strand specific detection is required.

- 20 Strand specific methods utilise additional nucleic acid reaction components to monitor the progress of amplification reactions. These methods use fluorescence resonance transfer (FRET) as the basis of detection. One or more nucleic acid probes are labelled with fluorescent molecules, a reporter molecule and a quencher molecule. The reporter molecule is
- 25 excited with a specific wavelength of light for which it will normally exhibit a fluorescence emission wavelength. The quencher molecule is also excited at this wavelength such that it can accept the emission energy of the reporter molecule by resonance transfer when they are in close proximity (e.g. on the same, or a neighbouring molecule). The basis of FRET
- 30 detection is to monitor the changes at reporter and quencher emission wavelengths. There are two types of FRET probes, those using hydrolysis of nucleic acid probes to separate reporter from quencher, and those using hybridisation to alter the spatial relationship of reporter and quencher molecules.
- 35

Hydrolysis probes are commercially available as TaqMan™ probes. These consist of DNA oligonucleotides that are

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labelled with reporter and quencher molecules. The probes are designed to bind to a specific region on one strand of a PCR product. Following annealing of the PCR primer to this strand, Taq enzyme extends the DNA with 5' to 3' polymerase activity. Taq enzyme also exhibits 5' to 3' exonuclease activity. TaqMan™ probes are protected at the 3' end by phosphorylation to prevent them from priming Taq extension. If the TaqMan™ probe is hybridised to the product strand than an extending Taq molecule may also hydrolyse the probe, liberating the reporter from quencher as the basis of detection.

The fact that signal generation is dependent upon the occurrence of probe hydrolysis reactions means that there is a time penalty associated with this method. This means that such techniques are not very compatible with rapid PCR methods which are becoming more prominent with the development of rapid hot air thermal cyclers such as the RapidCycler™ and LightCycler™ from Idaho Technologies Inc. Other rapid PCR devices are described for example in co-pending British Patent Application Nos. 9625442.0 and 9716052.7. The merits of rapid cycling over conventional thermal cycling have been reported elsewhere. Such techniques are particularly useful for example in detection systems for biological warfare where speed of result is important if loss of life or serious injury is to be avoided.

Furthermore, hydrolysis probes do not provide information with regard to hysteresis of melting since signal generation is dependant upon hydrolysis of the probe rather than the melt temperature of the amplicon.

Hybridisation probes are available in a number of guises. Molecular beacons are oligonucleotides that have complementary 5' and 3' sequences such that they form hairpin loops. Terminal fluorescent labels are in close proximity for FRET to occur when the hairpin structure is formed. Following

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hybridisation of molecular beacons to a complementary sequence the fluorescent labels are separated, so FRET does not occur, as the basis of detection. Pairs of labelled oligonucleotides may also be used. These hybridise in close
5 proximity on a PCR product strand bringing reporter and quencher molecules together so that FRET can occur. Enhanced FRET is the basis of detection. Variants of this type include using a labelled amplification primer with a single adjacent probe.

10 The applicants have developed a strand specific system for detecting the presence of particular nucleic acid sequences.

The invention provides a method for detecting the presence of
15 a target nucleic acid sequence in a sample, said method comprising

- (a) subjecting the sample to conditions under which said nucleic acid sequence becomes single stranded;
- 20 (b) applying to a sample suspected of containing said sequence an intercalating dye and a probe which is able to hybridise with said target sequence, said probe comprising a quencher molecule able to absorb fluorescence from said intercalating dye, under conditions in which the probe will hybridise with
25 said target sequence, and
- (c) observing changes in fluorescence in said sample.

As the probe hybridises to the target sequence, intercalating dye is trapped between the strands. In general, this would
30 increase the fluorescence at the wavelength associated with the dye. However, the quencher molecule is able to accept emission energy from the dye by means of FRET and so it emits fluorescence at its characteristic wavelength. Increase in fluorescence from the quencher molecule, which is of a
35 different wavelength to that of the dye, will indicate binding of the probe.

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Preferably, the fluorescence of both the dye and the quencher molecule are monitored and the relationship between the emissions calculated.

5 Suitable quencher molecules are rhodamine dyes or other dyes such as Cy5. These may be attached to the probe in a conventional manner. The position of the quencher along the probe is immaterial although in general, they will be positioned at an end region of the probe.

10

Intercalating dyes are well known in the art. They include for example Sybrgreen.

15 The use of an intercalating dye and a probe which is singly labelled is advantageous in that these components are much more economical than other assays in which doubly labelled probes are required. Furthermore the method of the invention is extremely versatile in its applications.

20 For example, it may be used in the course of an amplification reaction in order to monitor the progress of the amplification reaction and to provide a means for quantitating the amount of target sequence present in the sample. For this purpose, the amplification reaction is carried out in the presence of the
25 probe and the intercalating dye. During each cycle, amplicon strands containing the target sequence bind to probe and thereby generate a quencher signal. As the amount of amplicon in the sample increases, so the quencher signal will increase, providing a strand specific measure to complement
30 the generic DNA information provided by measuring fluorescence from the dye.

The probe may be designed such that it is hydrolysed by the DNA polymerase used in the amplification reaction thereby
35 releasing the quencher molecule. This provides a cumulative signal, with the amount of free quencher molecule present in the system increasing with each cycle. A cumulative signal of this type may be particularly preferred where the amount of

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target sequence is to be quantified. However, it is not necessary in this assay for the probe to be consumed in this way as the signal does not depend solely upon the dissociation of the probe.

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Where speed of reaction is of the greatest importance, for example in rapid PCR, it may be preferable that the probe is designed such that during the extension phase of the amplification reaction, the probe is released intact from the target sequence and so may take part again in the reaction. This may be achieved by using a 5'-3' exonuclease lacking enzyme such as Stoffer fragment of Taq or Pwo.

10

When used in this way, it is important to ensure that the probe is not extended during the extension phase of the reaction. Therefore, the 3' end of the probe is blocked, suitably by phosphorylation.

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The data generated in this way can be interpreted in various ways. For example, the emissions from the dye can be used in order to monitor the bulk rise in nucleic acid in the sample and this can be compared to the strand specific amplification, as measured by the relationship between the quencher and dye signals.

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Thus, a preferred embodiment of the invention comprises a method for detecting nucleic acid amplification comprising: performing nucleic acid amplification on a target polynucleotide in the presence of (a) a nucleic acid polymerase (b) at least one primer capable of hybridising to said target polynucleotide, (c) a fluorescent intercalating dye and (d) an oligonucleotide probe which is capable of binding to said target polynucleotide sequence and which contains a quencher molecule which is capable of absorbing fluorescence from the said dye; and monitoring changes in fluorescence during the amplification reaction.

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The amplification is suitably carried out using a pair of primers which are designed such that only the target nucleotide sequence within a DNA strand is amplified as is well understood in the art. The nucleic acid polymerase is suitably a thermostable polymerase such as Taq polymerase.

Suitable conditions under which the amplification reaction can be carried out are well known in the art. The optimum conditions may be variable in each case depending upon the particular amplicon involved, the nature of the primers used and the enzymes employed. The optimum conditions may be determined in each case by the skilled person. Typical denaturation temperatures are of the order of 95°C, typical annealing temperatures are of the order of 55°C and extension temperatures are of the order of 72°C.

In an alternative embodiment, the method of the invention can be used in hybridisation assays. By monitoring changes in the fluorescence over different temperatures, the temperature at which the probe separates or "melts" from the target sequence can be determined. This can be extremely useful in for example, to detect and if desired also to quantitate, polymorphisms and/or allelic variation in genetic diagnosis. The hysteresis of melting of the probe will be different if the target sequence varies by only one base pair. Thus where a sample contains only a single allelic variant, the temperature of melting of the probe will be a particular value which will be different from that found in a sample which contains only another allelic variant. A sample containing both allelic variants which show two melting points corresponding to each of the allelic variants.

This embodiment can be effected in conjunction with amplification reactions such as the PCR reaction mentioned above.

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Further aspects of the invention include kits for use in the method of the invention. These kits will contain a probe specific for a target nucleotide sequence which contains a quencher molecule. Additionally, they may contain an
5 intercalating dye which is compatible with said quencher molecule. Other potential components of the kit include reagents used in amplification reactions such as DNA polymerase.

10 The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 shows diagrammatically the interactions which are
15 utilised in the process of the invention; and

Figure 2 illustrates stages during an amplification reaction in accordance with the invention.

20 Figure 1A illustrates the action of an intercalating dye (1) which is in the presence of single stranded DNA (2), as would be found during the melt phase of a PCR reaction. The dye attaches to the DNA strands and fluoresce at a certain level. However, when the DNA becomes double stranded (3), the dye is
25 concentrated and the fluorescence increases significantly. This increase in fluorescence can be used to detect the formation of double stranded DNA. The fluorescence of the dye will be at a particular wavelength, for example in the green region of the spectrum.

30 The effect of intercalating dye (1) on a probe (4) in accordance with the invention is illustrated in Figure 1C. Some dye will bind to the nucleotides of the probe and will fluoresce at the background level. However, as a result of
35 FRET, some energy will pass to the quencher molecule (5) as indicated by the arrow and so this molecule will also fluoresce but at a different wavelength to that of the dye, for example, in the red region of the spectrum.

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- When the probe hybridises with a single stranded target sequence as illustrated in Figure 1D, any increase in the fluorescent energy from the dye passes to the quencher molecule (5) which thus fluoresces at a higher level. Increase in the fluorescence of the quencher molecule will thus be indicative of hybridisation of the probe to the target sequence. Thus by measuring the increase in fluorescence of the quencher molecule, for example as the temperature decreases, the point at which hybridisation occurs can be detected. Similarly, a decrease in quencher fluorescence will occur as the temperature increases at the temperature at which the probe melts from the target sequence. This will vary depending upon the hybridisation characteristics of the probe and the target sequence. For example, a probe which is completely complementary to a target sequence will melt at a different temperature to a probe which hybridises with the target sequence but contains one or more mismatches.
- Figure 2 illustrates how the method of the invention can be employed in amplification reactions such as the PCR reaction. Probe (5) will hybridise to single stranded DNA in conjunction with the intercalating dye and thus generate an increased quencher signal (Figure 2A). This will occur during the annealing phase of the cycle. As the amount of target sequence increases as a result of the amplification, the signal generated during the annealing phase by the quencher molecule will also increase.
- During the extension phase, the probe is removed from the target sequence either by hydrolysis or, as illustrated, because it is displaced by the DNA polymerase. At this point, the quencher signal decreases although the signal from the dye (1) will be enhanced, again indicative of the increase in the amount of target sequence.

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By monitoring the progress of the amplification reaction in this manner, the quantity of target sequence present in the original sample can be quantitated.

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Claims

- 5
1. A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising
- 10 (a) subjecting the sample to conditions under which said nucleic acid sequence becomes single stranded;
- (b) applying to a sample suspected of containing said sequence an intercalating dye and a probe which is able to hybridise with said target sequence, said probe comprising a quencher molecule able to absorb fluorescence from said intercalating dye, under conditions in which the probe will hybridise with
- 15 said target sequence, and
- (c) observing changes in fluorescence in said sample.
2. A method according to claim 1 wherein the fluorescence from both the dye and the quencher molecule are monitored and the relationship between the emissions calculated.
- 20
3. A method according to claim 1 or claim 2 wherein the quencher molecule is a rhodamine dye or Cy5.
- 25
4. A method according to any one of the preceding claims wherein the quencher molecule is attached at an end region of the probe.
- 30
5. A method according to any one of the preceding claims which is carried out concurrently with an amplification reaction.
- 35
6. A method according to claim 5 wherein an amplification reaction is carried out in the presence of the probe and the intercalating dye and the change in fluorescence is monitored throughout as the amplification reaction proceeds.

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7. A method according to claim 6 whereby fluorescence measurements are used to quantitate the amount of target nucleic acid sequence is present in the sample.
- 5 8. A method according to any one of claims 5 to 7 wherein the probe is designed such that it is hydrolysed by the DNA polymerase used in the amplification reaction.
9. A method according to any one of claims 5 to 7 wherein
10 the probe is released intact from the target sequence.
10. A method for detecting nucleic acid amplification comprising:
performing nucleic acid amplification on a target
15 polynucleotide in the presence of (a) a nucleic acid polymerase (b) at least one primer capable of hybridising to said target polynucleotide, (c) a fluorescent intercalating dye and (d) an oligonucleotide probe which is capable of
20 binding to said target polynucleotide sequence and which contains a quencher molecule which is capable of absorbing fluorescence from the said dye; and monitoring changes in fluorescence during the amplification reaction.
11. A method according to claim 10 wherein the amplification
25 is suitably carried out using a pair of amplification primers.
12. A method according to claim 10 or claim 11 wherein the nucleic acid polymerase is suitably a thermostable polymerase.
- 30 13. A method according to any one of the preceding claims wherein the temperature is altered and the fluorescence used to determine the temperature at which the probe hybridises with said target sequence.
- 35 14. A method according to claim 13 which is used to detect allelic variation or a polymorphism in a target sequence.

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17. A kit for use in the method according to any one of the preceding claims, which kit comprises a probe specific for a target nucleotide sequence which contains a quencher molecule, and an intercalating dye which is compatible with said quencher molecule.

18. A kit according to claim 17 which further comprises one or more reagents used in an amplification reaction.

19. A probe for use in a method according to any one of the preceding claims which comprises a sequence which will hybridise with a target nucleotide sequence and a quencher molecule.

5

Abstract

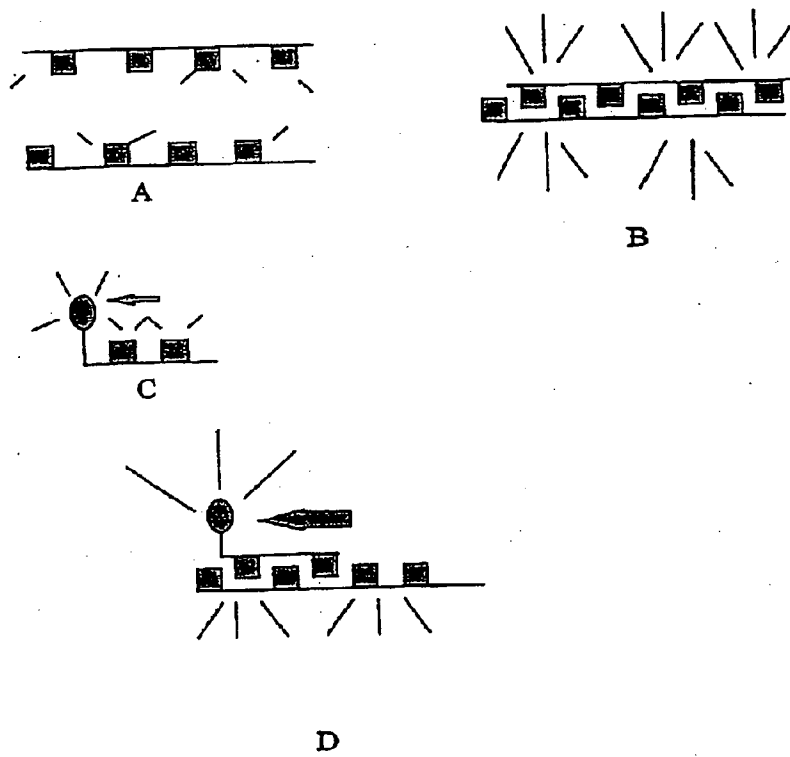
A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising

- 10 (a) subjecting the sample to conditions under which said nucleic acid sequence becomes single stranded;
- (b) applying to a sample suspected of containing said sequence an intercalating dye and a probe which is able to hybridise with said target sequence, said probe comprising a quencher
- 15 molecule able to absorb fluorescence from said intercalating dye, under conditions in which the probe will hybridise with said target sequence, and
- (c) observing changes in fluorescence in said sample.
- 20 This method can be used for example to monitor amplification reactions such as PCR reactions, such that the amount of target sequence present in the sample may be determined. Additionally or alternatively, it may be used to generate melt hysteresis data for amplification monitoring or for detection
- 25 and quantitation of polymorphisms or allelic variation, and so is useful in genetic diagnosis.

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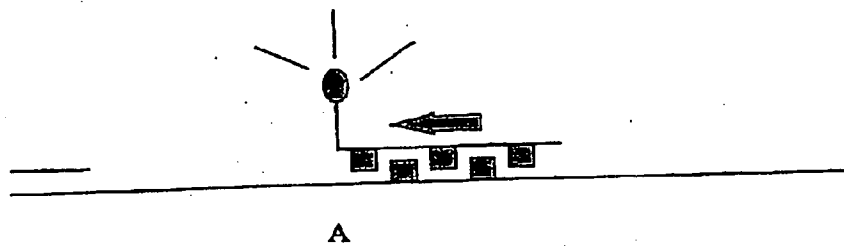
Figure 1



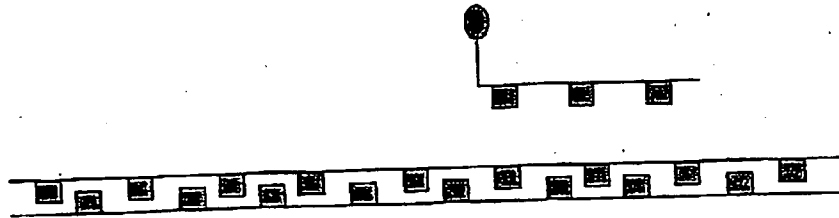
19

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Figure 2



A



B

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